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RESEARCH PAPER

Amiselimod, a novel sphingosine 1-phosphate receptor-1 modulator, has potent therapeutic efficacy for autoimmune diseases, with low bradycardia risk

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BACKGROUND AND PURPOSE

We conducted preclinical and clinical studies to examine the pharmacological, particularly cardiac, effects of amiselimod (MT-1303), a second-generation sphingosine 1-phosphate (S1P) receptor modulator, designed to reduce the bradycardia associated with fingolimod and other S1P receptor modulators.

EXPERIMENTAL APPROACH

The selectivity of the active metabolite amiselimod phosphate (amiselimod-P) for human S1P receptors and activation of G-protein-coupled inwardly rectifying K^+ (GIRK) channels in human atrial myocytes were assessed. Its cardiac distribution was determined in rats, and cardiovascular telemetry was assessed in monkeys. We also examined the pharmacokinetics, pharmacodynamics and safety of amiselimod in healthy humans.

KEY RESULTS

Amiselimod-P showed potent selectivity for $S1P_1$ and high selectivity for $S1P_5$ receptors, with minimal agonist activity for $S1P_4$ and no distinct agonist activity for $S1P_2$ or $S1P_3$ receptors and approximately five-fold weaker GIRK activation than fingolimod-P. After oral administration of amiselimod or fingolimod at 1 $mg \cdot kg^{-1}$, the concentration of amiselimod-P in rat heart tissue was lower than that of fingolimod-P, potentially contributing to the minimal cardiac effects of amiselimod. A telemetry study in monkeys confirmed that amiselimod did not affect heart rate or ECG parameters. In healthy human subjects, peripheral blood lymphocyte counts gradually reduced over the 21 day dosing period, with similar lymphocyte count profiles with the highest doses by day 21, and no clinically significant bradycardia observed on day 1 or during the study.

CONCLUSIONS AND IMPLICATIONS

Amiselimod exhibited potent therapeutic efficacy with minimal cardiac effects at the anticipated clinical dose and is unlikely to require dose titration.

Abbreviations

S1P, sphingosine 1-phosphate; RRMS, relapsing–remitting multiple sclerosis; GIRK, G-protein-activated inwardly rectifying potassium; HCMs, human cardiac myocytes; AE, adverse event

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Tables of Links

TARGETS	
GPCRs[a]	**Voltage-gated ion channels**[b]
$S1P_1$ receptor	G-protein-activated inward-rectifier K channel ($K_{ir}3.1$)
$S1P_2$ receptor	G-protein-activated inward-rectifier K channel ($K_{ir}3.2$)
$S1P_3$ receptor	G-protein-activated inward-rectifier K channel ($K_{ir}3.3$)
$S1P_4$ receptor	G-protein-activated inward-rectifier K channel ($K_{ir}3.4$)
$S1P_5$ receptor	

LIGANDS
Amiselimod
Sphingosine 1-phosphate
Fingolimod
Fingolimod phosphate

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 ([a,b]Alexander *et al.*, 2015a,b).

Introduction

Sphingosine 1-phosphate (S1P), a multi-functional lysophospholipid mediator, is generated from sphingosine by sphingosine kinases (SPHKs) and binds to five subtypes of G protein-coupled S1P receptors ($S1P_{1–5}$) (Alexander *et al.*, 2015a). S1P-driven signalling mediates a variety of cellular responses, including cell survival, regulation of cell proliferation, cell migration, cytoskeletal organization, immune cell trafficking, angiogenesis and tumour cell invasion. Mice lacking expression of $S1P_1$ receptors in lymphocytes show decreased trafficking of mature T cells from the thymus to the periphery and greatly reduced numbers of B cells in blood and lymph, indicating the essential role of S1P and $S1P_1$ receptors in lymphocyte egress from the thymus and secondary lymphoid organs (Allende *et al.*, 2004; Matloubian *et al.*, 2004).

S1P receptor modulators act as functional antagonists at lymphocytic $S1P_1$ receptors, inhibit $S1P_1$ receptor-dependent lymphocyte egress from the secondary lymphoid organs to the periphery and decrease numbers of circulating lymphocytes including auto-reactive T cells, resulting in immunomodulatory effects (Lo *et al.*, 2005; Chiba *et al.*, 2006). Several S1P receptor modulators have been developed for the treatment of various autoimmune diseases, and their efficacy was confirmed in clinical trials of many such agents for relapsing–remitting multiple sclerosis (RRMS), psoriasis and ulcerative colitis (Cohen *et al.*, 2010; Kappos *et al.*, 2010; Selmaj *et al.*, 2013; Vaclavkova *et al.*, 2014; Sandborn *et al.*, 2015). However, a transient reduction in heart rate with the first dose is a known effect of many S1P receptor modulators (Budde *et al.*, 2002; Schmouder *et al.*, 2006, Bigaud *et al.*, 2014). Currently, it is recommended that patients receiving fingolimod, the only approved agent in the class, are monitored for at least 6 h after the first dose (Jeffery *et al.*, 2011).

$S1P_3$ receptor agonism has been postulated to be the cause of bradycardia with S1P receptor ligands. This belief is based on animal data suggesting that the transient bradycardia produced by fingolimod may predominantly involve the $S1P_3$ receptor (Forrest *et al.*, 2004; Sanna *et al.*, 2004). However, in humans, a detailed analysis of cardiovascular tissue by *in situ* hybridization and immunohistochemistry has shown that $S1P_1$ receptor mRNA and protein are strongly expressed in human ventricular, septal and atrial cardiomyocytes, whereas $S1P_3$ receptor mRNA is weakly expressed (Mazurais *et al.*, 2002). Indeed, a study with the $S1P_{1/5}$ receptor-selective agonist siponimod (BAF312) induced rapid, transient (1 day) bradycardia in humans, although it lacked activity at $S1P_3$ receptors. The authors measured the activity of G-protein-activated inwardly rectifying potassium (GIRK) channels and speculated that the BAF312-mediated activation of GIRK channels in human atrial myocytes was a possible cellular mechanism for the bradycardia effects (Gergely *et al.*, 2012). Taken together, these results suggest that agonism of $S1P_1$, rather than $S1P_3$ receptors appears to play a major role in bradycardia via GIRK channel activation by S1P receptor agonists in humans (Gergely *et al.*, 2012, Bigaud *et al.*, 2014).

There are two types of S1P receptor modulators: i) prodrugs such as fingolimod converted to an active metabolite by SPHKs; and ii) direct-acting drugs such as ponesimod, siponimod and ozanimod (Bigaud *et al.*, 2014). Similar to fingolimod, amiselimod (MT-1303; 2-amino-2-{2-[4-(heptyloxy)-3-(trifluoromethyl)phenyl] ethyl} propan-1,3-diol hydrochloride) was designed as a prodrug S1P receptor modulator lacking $S1P_3$ receptor agonism to avoid bradycardia with the first dose. Amiselimod is converted to its active metabolite, (*S*)-amiselimod phosphate (amiselimod-P), by SPHKs (Figure 1). It functions as a highly selective $S1P_1$ receptor functional antagonist without activity at $S1P_3$ receptors. In addition to its lack of $S1P_3$ receptor agonist activity, amiselimod was designed to be converted to the active metabolite in human cells (*in vitro*) more slowly than fingolimod, because it was thought that its effect on the heart rate of humans may be ameliorated by the gradual increase in concentration

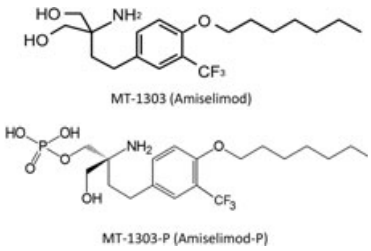

Figure 1
Chemical structures of amiselimod and amiselimod phosphate (amiselimod-P).

of its active metabolite in the heart following administration.

This study reports both preclinical and phase I clinical data for amiselimod. In preclinical studies, the pharmacological profile of amiselimod, especially with regard to its cardiac effects, was investigated. The phase I clinical study was conducted to characterize initial cardiac effects following amiselimod administration and the effects on peripheral blood lymphocyte counts. The pharmacokinetic and safety profiles of amiselimod were also investigated. It was hypothesized that amiselimod would have therapeutic potential for RRMS and possibly other autoimmune diseases, with little influence on heart rate and that our preclinical results would demonstrate minimal cardiac effects of amiselimod at its anticipated clinical dose.

Methods

Animals

All animal care and experimental protocols were approved by the ethics review committee for animal experimentation of Research Division Mitsubishi Tanabe Pharma. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath & Lilley, 2015). Six-week-old male SD rats were purchased from Charles River Japan (Yokohama, Japan). SD rats are widely used for pharmacokinetic analysis; moreover, these species and strain have previously been used in safety studies of amiselimod. Rats were housed two to three per stainless cage under conventional conditions. They were kept at a constant temperature of 23 ± 3°C and relative humidity of 30–70% under a 12 h light/dark cycle. Food and water were available *ad libitum*.

Tissue distribution in rats

Seven-week-old male SD rats (30 rats in total; weighing 208–231 g) were allocated to 10 experimental groups (n = 3 per experimental condition) using the simulated method to ensure all groups were comparable in terms of body weight. Rats were fasted for 15 h and then given (at 0 h), without anaesthesia, the allocated study drug (amiselimod or fingolimod) at a single oral dose of 1-mg·kg^{-1}. At the following times after administration: 2, 4, 8, 24 or 48 h, the rats were anaesthetized (three rats per time point and group) with isoflurane and killed by exsanguination from the inferior vena cava. Blood was collected and the hearts were removed. The rats killed at 24 or 48 h were given food at 8 h.

Blood samples were centrifuged to obtain plasma, and hearts were homogenized. After pretreatment of plasma and heart homogenates with solid-phase extraction, concentrations of unchanged compound and its phosphate metabolite were measured by LC–MS/MS (API 4000, AB SCIEX). Drug administration or tissue sampling and sample analysis were carried out by different researchers, unaware of the treatments.

The total duration of fasting (23 h) was used to exclude possible food effects (such as motility, blood flow and bile secretion) on the pharmacokinetics of the test drug. Moreover, in a previous drug disposition study using a radiolabelled compound, rats were fasted for about 24 h (unpublished data). Therefore, to compare the pharmacokinetics of the present study with the previous drug disposition study, the feeding condition was set to a similar duration. Because starvation for more than 24 h inflicts pain corresponding to category D established by our in-house Institutional Animal Care and Use Committee (followed by the Scientists Center for Animal Welfare), the fasting period was set at 23 h to minimize distress. This fasting period did not affect the conditions of the animals used in this study.

Cardiovascular telemetry in monkeys

These experiments were performed by LSI Medience Corporation (Tokyo, Japan) as a contract research organization under approval of the Institutional Animal Care and Use Committee of LSI Medience Corporation. LSI Medience Corporation was approved by the ethics review committee for animal experimentation of Research Division Mitsubishi Tanabe Pharma, and complied with the ARRIVE guidelines. This study was carried out in compliance with Good Laboratory Practice Standards in 2007–2008. Four male cynomolgus monkeys (*Macaca fascicularis*, 3–4 years old) were obtained from Japan Laboratory Animals Inc. (Breeder: NAFO-VANNY, Vietnam) and maintained at a temperature of 24.4–29.3°C with a relative humidity of 39.4–80.8% under a 12 h light/dark cycle. Food [pellet diet for experimental animals (CMK-2, CLEA Japan Inc.) supplied at 100 g per day] and water (membrane-filter-purified) were available *ad libitum*. During the study, the monkeys were housed singly to detect a slight decrease in heart rate reported by fingolimod administration (Food and Drug Administration, 2010) in a quiet atmosphere without interference from other monkeys in the same cage and to prevent injury and removal of measuring equipment by other monkeys.

The general condition of monkeys was checked daily. For all experiments, the animals showed no abnormalities by observation of clinical signs, body weight determination, tuberculin test or microbial examination of stools. A transmitter (TL11M2-D70-PCT, Data Sciences International Inc., New Brighton, MN, USA) was surgically implanted into the intra-abdominal cavity in male cynomolgus monkeys under anaesthesia with i.v. pentobarbital sodium at 30 mg·kg^{-1} (Nembutal, Abbott Labs., Chicago,

IL, USA). ECG electrodes were placed subcutaneously at the lateral part of the right thoracic region and left abdominal region, and the tip of the pressure catheter connected to the transmitter was fixed in the abdominal aorta through the femoral artery. Following surgery, the animals were injected i.m. with 0.01 $mg \cdot kg^{-1}$ buprenorphine hydrochloride (Lepetan injection, Otsuka Pharmaceutical Co., Tokyo, Japan). The transmitters remained *in situ* for the duration of the study. Heart rate, ECG and blood pressure were recorded with a telemetry data collection and analysis system (HEM, version 4.2, Notocord Systems, Croissy-sur-Seine, France). At least 1-month later and having confirmed that all four monkeys had recovered well, amiselimod was orally administered in a dose-ascending manner at dose levels of vehicle (0.5% HPMC), 0.3, 3 and 30 mg kg^{-1}, with a 6 day interval between vehicle and 0.3 $mg \cdot kg^{-1}$, and 13 or 14 day intervals between dose levels of 0.3, 3 and 30 $mg \cdot kg^{-1}$. Each monkey received all doses of amiselimod and vehicle. The heart rate was analysed before and 4, 8, 24, 48 and 72 h after each dose. The heart rate was automatically recorded for 30 s every 5 min. The mean of three data points was used for each evaluation time point (the intended time point and 5 min before and after the time point). The analyses were conducted under a standard operation procedure at LSI Medience by a researcher blinded to the drug profiles. At the end of the study, the monkeys with the transmitter were returned to LSI Medience Corporation under approval from the Institutional Animal Care and Use Committee of LSI Medience Corporation and were available for use in later studies.

Ca^{2+} mobilization assay

Human S1P receptor-expressing cells were loaded with 5$\mu mol \cdot L^{-1}$ Fura 2-AM (Dojindo Laboratories Corporation, Kumamoto, Japan) and 0.1% Pluronic F-127 (Life Technologies, Carlsbad, CA) in HBSS (Invitrogen Corporation, Waltham, CA, USA), pH 7.4, containing 0.1% fatty acid-free BSA (Sigma-Aldrich Inc., St. Louis, MO, USA) and 1.25 $mmol \cdot L^{-1}$ probenecid (Sigma-Aldrich Inc.), then incubated in a CO_2 incubator for 60–120 min. After loading, cells were washed with PBS and detached from the flask with trypsin–EDTA. Then, the cells were washed and seeded in 96-well clear-bottom black plates (3×10^4 cells in 100 μL, per well). Test substance solutions in 25 μL aliquots per well were added, and changes in fluorescence intensity resulting from induction of intracellular Ca^{2+} mobilization were measured for 210 s using a Functional Drug Screening System 3000 (FDSS3000, Hamamatsu Photonics K.K., Hamamatsu, Japan). The ratio of fluorescence intensities at 540 nm on excitation at wavelengths 340 and 380 nm was used as an index of the change in intracellular Ca^{2+} mobilization. For the human $S1P_4$ receptor-expressing cells, the Calcium Kit II-Fura 2 (Dojindo Laboratories Corporation) was used to load the cells with Fura2-AM. The average was taken from three independent experiments with each experiment run in duplicate and expressed as percentage response activity to the maximum response to S1P. Therefore, six wells were measured at each time point.

Human GIRK channel assay in human atrial myocytes

All human tissue samples were obtained with full informed consent and after ethical review in accordance with Tulane University School of Medicine Institutional guidelines. A specimen of human atrium was obtained from a patient undergoing cardiac surgery, and atrial myocytes were isolated according to a previously described method (Crumb *et al.*, 1995). GIRK currents were measured using the whole-cell variant of the patch clamp method at a temperature of 37 ± 1°C. The external (bathing the cell) solution had an ionic composition of 120 $mmol \cdot L^{-1}$ NaCl, 20 $mmol \cdot L^{-1}$ KCl, 2 $mmol \cdot L^{-1}$ $CaCl_2$, 1 $mmol \cdot L^{-1}$ $MgCl_2$, 11 $mmol \cdot L^{-1}$ dextrose and 10 $mmol \cdot L^{-1}$ HEPES (pH 7.4). The internal (pipette) solution had an ionic composition of 100-$mmol \cdot L^{-1}$ potassium aspartate, 40 $mmol \cdot L^{-1}$ KCl, 5.0-$mmol \cdot L^{-1}$ magnesium ATP, 2.0 $mmol \cdot L^{-1}$ EGTA, 0.01 $mmol \cdot L^{-1}$ GTP-Tris and 10 $mmol \cdot L^{-1}$ HEPES (pH 7.2). In each cell, the GIRK current elicited by rapid superfusion with 20 $\mu mol \cdot L^{-1}$ carbachol was measured by a current pulse to −100 mV from a holding potential of −90 mV. After a washout period, the test compound was superfused until an apparent steady-state effect was observed at each concentration. The test concentration was progressively increased in a step-wise manner. Peak inward current induced by the test compound was normalized to the peak amplitude of current evoked by 20 $\mu mol \cdot L^{-1}$ carbachol. The GIRK current was measured in four independent myocytes per treatment condition. The analyses were conducted by a researcher blinded to the drug profiles.

In vitro phosphorylation in human cells

HEK293 cells or primary human cardiac myocytes (HCMs) were plated on 48-well culture plates (0.2 mL per well) at a density of 1.25×10^5 $cells \cdot mL^{-1}$ and cultured overnight in a CO_2 incubator. The next day, amiselimod or fingolimod was added to individual wells at final concentration of 100-$nmol \cdot L^{-1}$, and the supernatants were obtained at 3, 6 and 12 h after the addition of test substances. The concentrations of amiselimod-P and fingolimod-P in the supernatants were measured by LC–MS/MS (API 4000, AB SCIEX, Framingham, MA, USA) after pretreatment with solid-phase extraction. *In vitro* phosphorylation was measured in three independent cultures per experimental condition. The analyses were conducted by an independent researcher who was not involved in performing the assay.

Phase I multiple ascending dose trial

All subjects provided written informed consent before study participation. The study was conducted through Simbec Research Limited (Wales, UK) after approval by the South East Wales Research Ethics Committees. The study was a phase I, randomized, parallel, double-blind, placebo-controlled, ascending, multiple-dose, pharmacokinetic, pharmacodynamic, safety and tolerability study of amiselimod in healthy subjects using four ascending dose levels of amiselimod (0.125, 0.25, 0.5 and 0.75 mg amiselimod, 10 subjects at each dose) and a placebo control (two subjects for each dose level and two subjects for loading dose regimen; $n = 10$). After a screening, treatment with amiselimod or matching placebo

was over 21 days in the fed state. Follow-up visits were on days 28, 35, 42, 56 and 70. The study population comprised healthy male subjects between the ages of 18 and 45 years inclusive. Fifty subjects completed the study.

The primary objective of the study was to evaluate the safety and tolerability of multiple oral doses of amiselimod and to determine the pharmacokinetic profiles of amiselimod and its active metabolite amiselimod-P in healthy volunteers. Secondary objectives included characterization of amiselimod pharmacodynamics and the effect on lymphocyte counts and lymphocyte recovery, as well as the magnitude of any changes in heart rate and pulmonary function.

Heart rate was assessed using 24 h 12-lead Holter monitoring at screening, on days −1, 1, 7, 14, 21 and 22. If the hourly heart rate parameters were zero because of signal loss or any form of artefact, they were treated as missing in the calculation of derived parameters. Daily (24 h) and hourly summary parameters and annotation from Holter ECG recordings were provided by CardioAnalytics Ltd (Plymouth, UK). To ensure safety, telemetry was also used for real-time monitoring on day 1. Absolute lymphocyte counts were assessed at screening and on days −2, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 16, 18, 20, 21, 22, 23, 24, 25, 28, 35, 42, 56 and 70. Adverse events (AEs) were recorded throughout the study.

Further assessments included routine laboratory safety (biochemistry, haematology, coagulation and urinalysis), physical examination, body weight, vital signs (supine blood pressure and pulse, oral temperature), 12-lead ECG, pulmonary function (forced expiratory volume in 1 s, forced vital capacity, forced expiratory flow between 25 and 75% of vital capacity and diffusing capacity of lung for carbon monoxide), chest X-ray, echocardiography, ophthalmological examination, visual acuity and optical coherence tomography, pulse oximetry, drugs of abuse and serology.

Pharmacokinetic assessment

Venous blood samples (4mL) were collected into potassium-EDTA polypropylene tubes on days 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 16, 18, 20, 21, 22, 23, 24, 25, 28, 35, 42, 56 and 70. Urine samples were collected into pre-weighed polypropylene urine jars during each interval, on days 1 (pre-dose, 0–6, 6–12 and 12–24 h), 21 (480–486, 486–492 and 492–504 h), 22, 23 and 24. Amiselimod and amiselimod-P were quantified in plasma and urine using a validated specific LC/MS/MS bioanalytical method for pharmacokinetic assessments. The lower limit of quantitation was 0.05 $ng \cdot mL^{-1}$ for plasma and urine. Detailed methods are described in the Supplemental materials.

Data analysis

Nonclinical studies. The data and statistical analysis in this study comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). For the Ca^{2+} mobilization assay, the geometric mean and 95% confidence interval (CI) of the EC_{50} value of each test substance were calculated by nonlinear regression analysis. The analyses for the Ca^{2+} mobilization assay and monkey telemetry were conducted using SAS® Version 8.2 or later (SAS Institute Inc., Cary, NC, USA). Plasma and heart drug concentrations in rats were analysed by a non-compartmental model using the pharmacokinetics analysis program WinNonlin version 6.3 (Pharsight Corporation, Mountain View, CA, USA) to calculate the pharmacokinetic parameters. GIRK channel study and monkey telemetry studies were conducted by Zenas Technologies LLC (New Orleans, LA, USA) and LSI Medience Corporation, respectively, and the analyses of these studies were also performed by each contract research organization.

Monkey telemetry data analysis. Statistical analyses were performed using Bartlett's test for homogeneity of variance. When the variances were homogeneous, the data were analysed by parametric Dunnett's mean multiple comparison test. When the variances were not homogeneous, the data were analysed by non-parametric Dunnett's mean multiple comparison test. Bartlett's test was conducted at a significance level of 5%, and the other tests were conducted at a two-tailed significance level of 5%.

Clinical study. As this was a typical phase I study, no formal statistical assumptions were made. Mean hourly heart rate was calculated by a linear mixed model with dose-by-hour and dose-by-hour-by-visit as fixed effects and pre-dose mean heart rate as a covariate. First-order autoregressive type was used for covariance structure of the residual to evaluate overall heart rate profile. Lowest mean hourly heart rate from dosing time to 12 h post-dosing was calculated as the nadir (1–12 h). The nadir (1–12 h) was analysed using ANCOVA with dose as a fixed effect and the value on day −1 as a covariate. Differences in least squares (LS) means and 95% CI were calculated for each comparison of active dose versus placebo. These analyses were conducted using SAS® Version 9.1.3 and 9.2 (SAS Institute Inc.). Plasma concentrations were analysed by a non-compartmental model using WinNonlin version 5.2.1 or later (Pharsight Corporation) to calculate the pharmacokinetic parameters. All analyses were performed by Simbec Research Limited (Wales, UK).

Materials and cells

Amiselimod (MT-1303), fingolimod, amiselimod-P and fingolimod-P were provided by Mitsubishi Tanabe Pharma Corporation (Osaka, Japan). S1P was obtained from Avanti Polar Lipids (Alabaster, AL, USA). Human S1P receptor ($S1P_{1-5}$)-expressing cells were purchased from Chemicon International, Inc. (Temecula, CA, USA). Cells were cultured in DMEM containing 10% FBS, non-essential amino acids, 10 $mmol \cdot L^{-1}$ HEPES, 100 $U \cdot mL^{-1}$ penicillin, 100 $\mu g \cdot mL^{-1}$ streptomycin and 250 $\mu g \cdot mL^{-1}$ geneticin. Primary human cardiac myocytes (HCMs; ScienCell Research Laboratories, Carlsbad, CA, USA) were cultured in cardiac myocyte medium (ScienCell Research Laboratories) according to the supplier's instructions.

Results

Selectivity towards S1P receptors

The effects of amiselimod-P, fingolimod-P and S1P on human S1P receptors ($S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$ and $S1P_5$) were examined using an intracellular Ca^{2+} mobilization assay to

Table 1

Agonist activity of S1P, fingolimod-P, amiselimod-P and amiselimod on human S1P receptors

Compound	EC_{50} values (95% CI), nmol·L^{-1}				
	$S1P_1$	$S1P_2$	$S1P_3$	$S1P_4$	$S1P_5$
S1P	0.18 (0.070–0.45)	2.3 (0.43–12.3)	0.16 (0.036–0.69)	82.7 (46.6–146.9)	0.50 (0.10–2.5)
Fingolimod-P	0.087 (0.061–0.12)	>1000[a]	5.1 (2.2–12)	66.3 (45.0–97.9)	0.24 (0.047–1.2)
Amiselimod-P	0.075 (0.047–0.12)	>10 000	>10 000	122.3 (102.5–146.1)	0.47 (0.11–1.9)
Amiselimod	>1000	NT	NT	NT	NT

EC_{50} values were calculated by nonlinear regression analysis (three independent experiments with each experiment run in duplicate).
[a]Fingolimod-P at 10 000 nmol·L^{-1} induced intracellular Ca^{2+} mobilization by 14.2% (percentage of the maximum response of S1P).
NT – not tested.

clarify their selectivity. The amiselimod prodrug (up to 1000 nmol·L^{-1}) showed no agonist activity at $S1P_1$ receptors. The active metabolite amiselimod-P showed agonist activity at $S1P_1$ (EC_{50} 75pmol·L^{-1}) and was more potent than at $S1P_4$ and $S1P_5$ receptors but had no distinct agonist activity at $S1P_2$ or $S1P_3$ receptors (Table 1). In contrast, S1P showed agonist activity at all five human S1P receptors, and fingolimod-P exhibited agonist activity at $S1P_1$, $S1P_3$, $S1P_4$ and $S1P_5$, but not $S1P_2$ receptors Of note, the differences in agonist activity for $S1P_4$ and $S1P_5$ receptors between fingolimod-P and amiselimod-P (Table 1) were only two-fold.

Effect of amiselimod-P on activation of GIRK channels in human atrial myocytes

We characterized the effects of S1P, amiselimod-P and fingolimod-P on the currents through GIRK channels, recorded from acutely isolated human atrial myocytes. The natural ligand S1P potently activated GIRK channels with an EC_{50} value of 1.9 nmol·L^{-1}, reaching more than 90% of the maximal current increase that was evoked by carbachol. Amiselimod-P, fingolimod-P and S1P increased GIRK current amplitude in a concentration-dependent manner, with EC_{50} values of 41.6, 8.5 and 1.9 nmol·L^{-1} respectively (Figure 2 and Figure S1). Amiselimod-P was the least potent compound, having an EC_{50} value approximately 20 times greater than S1P and five times greater than that of fingolimod-P.

Effect of amiselimod-P on heart rate or ECG parameters on monkeys

A reduction of heart rate was induced via different S1P receptor subtypes in rodents and humans by S1P receptor modulators (Bigaud *et al.*, 2014). Among non-rodents, administration of fingolimod had been confirmed to decrease heart rate in monkeys (Food and Drug Administration, 2010). Therefore, the effect of amiselimod on heart rate monitored by telemetry in conscious monkeys was examined. Amiselimod had no effects on heart rate or ECG parameters, including PR interval, QRS duration, QT interval and QTc at doses up to 30 mg·kg^{-1} (Figure S2). Furthermore, amiselimod did not affect blood pressure at doses up to 30 mg·kg^{-1}.

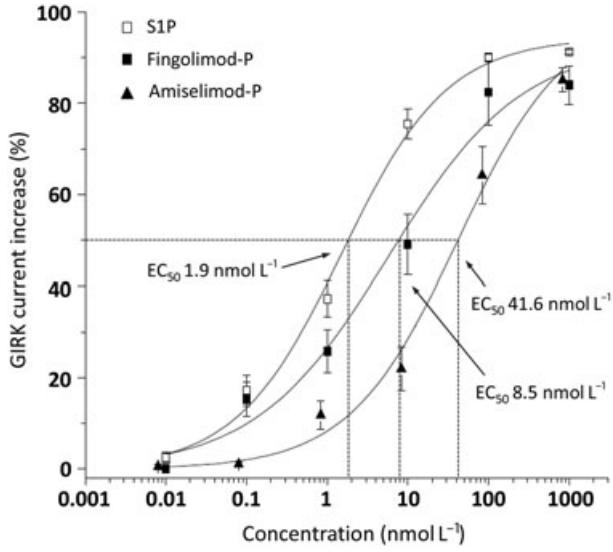

Figure 2

Effects of amiselimod-P, fingolimod-P and S1P on human GIRK currents. GIRK currents recorded from acutely isolated human atrial myocytes were measured using the whole-cell variant of the patch clamp method. Values were normalized to the current increase evoked by 20 μmol·L^{-1} carbachol. Results are expressed as the mean ± SEM (n = 4 independent measurements from different myocytes).

In vitro phosphorylation of metabolites in human cells

We compared the rate of conversion of amiselimod to amiselimod-P with that of fingolimod to fingolimod-P in human cells *in vitro*. Amiselimod or fingolimod was added to HEK293 cells or primary HCMs, and the concentrations of the phosphorylated active metabolites in supernatants were measured over time. Fingolimod-P was first detected in the supernatant of HEK293 cells and HCMs after 3 h of incubation and increased time-dependently (Figure 3). In contrast, amiselimod-P was first detected in both supernatants after 6 h of incubation, and the concentrations of amiselimod-P after 12 h reached approximately one-half of those of fingolimod-P (Figure 3). Thus, amiselimod was converted

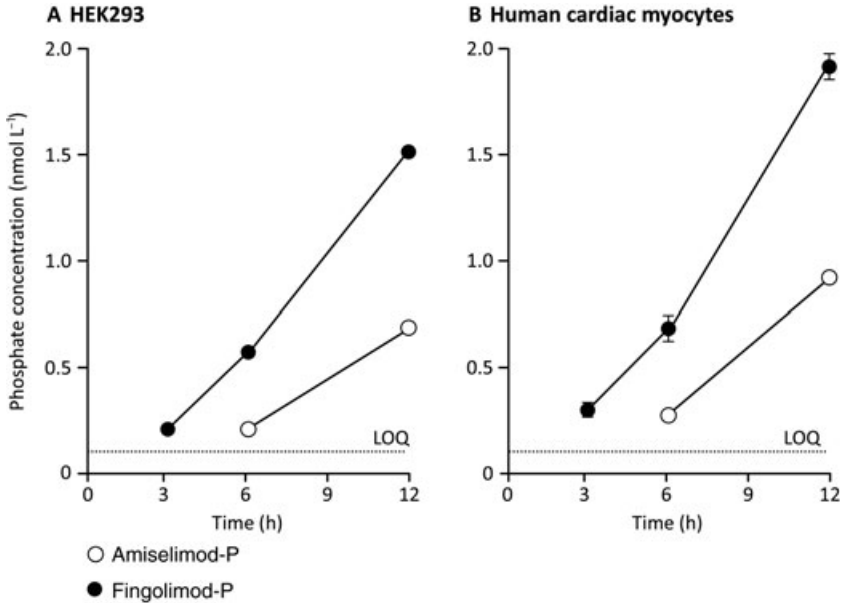

Figure 3

Conversion to amiselimod-P or fingolimod-P in human cells *in vitro*. (A) HEK293 cells or (B) HCMs were plated onto 48-well culture plates, cultured overnight and then incubated with 100 nmol·L^{-1} of amiselimod or fingolimod for 3–12 h. Supernatants were collected, and the concentrations of amiselimod-P or fingolimod-P were measured by LC–MS/MS. Results are expressed as the mean ± SEM (n = 3 independent cultures per experimental condition). LOQ: the lower limit of quantification.

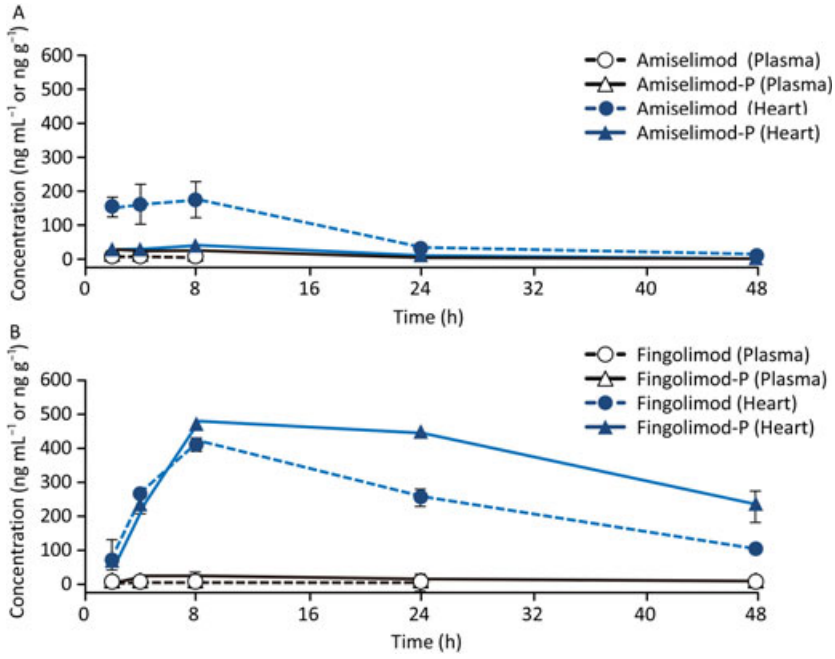

Figure 4

Distribution of amiselimod-P or fingolimod-P to cardiac tissue and to plasma, after oral administration to rats. (A) Amiselimod or (B) fingolimod at 1 mg·kg^{-1} was orally administered to SD rats, and plasma and hearts were collected. The concentrations of amiselimod/amiselimod-P or fingolimod/fingolimod-P in plasma and heart were measured by LC–MS/MS. Results are expressed as the mean ± SD (n = 3 at each time point per treatment group).

into amiselimod-P in human cells more slowly than fingolimod was converted into fingolimod-P.

Distribution to cardiac tissue in rats

The concentrations of unchanged drugs and active metabolites in hearts and plasma were measured over time following a single oral administration of amiselimod or fingolimod at 1-mg·kg^{-1}. The concentration-time profiles of amiselimod, amiselimod-P, fingolimod and fingolimod-P in rat plasma and heart homogenates are shown in Figure 4. The plasma AUC_{0-last} value (525 ng·h·mL^{-1}) for amiselimod-P was comparable with the plasma AUC_{0-last} value (556 ng·h·mL^{-1}) for fingolimod-P, whereas the heart AUC_{0-last} value (846-ng·h·g^{-1}) for amiselimod-P was approximately 1/20 of the heart AUC_{0-last} value (17 182 ng·h·g^{-1}) for fingolimod-P. The C_{max} value (42 ng·g^{-1}) of the heart amiselimod-P concentrations was approximately 1/10 of the C_{max} value (479-ng·g^{-1}) of the heart fingolimod-P concentrations (Table S1). Thus, in rats, the concentration of amiselimod-P in the heart was relatively lower than fingolimod-P.

Effect on peripheral lymphocyte counts in humans

A dose-dependent reduction in lymphocyte counts was observed in healthy subjects, receiving between 0.125 and 0.5 mg amiselimod, although lymphocyte count profiles were similar between 0.5 and 0.75 mg amiselimod on day 21, suggesting that the effects on lymphocytes might have reached or were approaching a plateau at the higher dose levels (Figure 5). Trough lymphocyte counts on day 21 decreased by 66 and 60% at doses of 0.5 and 0.75 mg respectively. There was a gradual reduction in lymphocyte counts over the 21 day dosing period, with values appearing to stabilize around day 14. The reduction was consistent across the 70 day sampling period, reflecting the long $t_{1/2}$ of amiselimod-P, with a gradual recovery in lymphocyte counts

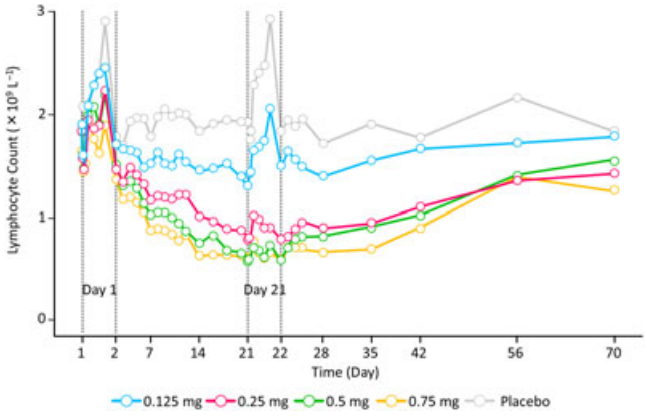

Figure 5

Mean lymphocyte count (×10^9 cells·L^{-1}) time profiles in healthy subjects. Absolute lymphocyte counts were assessed at screening and on days −2, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 16, 18, 20, 21, 22, 23, 24, 25, 28, 35, 42, 56 and 70 of the phase I clinical study (amiselimod: n = 10 per dose; placebo: n = 10). Amiselimod or placebo was administered on day 1.

observed from approximately day 21 (when dosing was stopped) to final follow-up on day 70.

Effect on heart rate after the first dose in humans

The mean hourly heart rate curves for all groups on day 1 are shown in Figure 6A. There was no obvious difference in each group. At the 0.5 mg dose of amiselimod, which was the dose with the maximum pharmacodynamic effect on lymphocyte counts in this study design (Figure 5), no clear clinical significant decrease in heart rate was observed during the treatment period (Figure 6B). A similar heart rate pattern was observed in the 0.75 mg dose group (Figure S3). Statistical analysis of the nadir (1–12 h) showed a slight decrease in heart rate on day 1 compared with placebo as the dose increased [LS mean 62.213, LS mean difference −2.135 (95% CI: −6.515 to 2.245) beats per minute (bpm) for 0.5 mg; LS mean 61.952, LS mean difference −2.396 (95% CI: −6.759 to 1.968) bpm for 0.75 mg by nadir (1–12 h)]. However, no significant decrease was detected up to the 0.75 mg dose.

ECG Holter transient AV conduction abnormalities were reported in five subjects subsequent to the administration of amiselimod. Mobitz Type 1 blocks (Wenckebach) were observed in one subject on day 2 and one subject on day 22, following 0.125 mg amiselimod, and two subjects on day 1 following 0.75 mg amiselimod. However, for one of the subjects in the 0.75 mg amiselimod group, these blocks were also observed at baseline prior to administration of amiselimod. First-degree AV-block was observed in one subject on day 22 following 0.125 mg amiselimod. In the placebo group, one subject experienced first-degree AV-block on days 2 and 7 and Mobitz Type 1 block on day 21. These subjects were asymptomatic, and the arrhythmias were considered not clinically significant by the investigator. There was no dose-dependent trend observed in AV conduction abnormalities.

No subject who received amiselimod had out of range QTc intervals that were >500 ms, or an increase in QTcF or QTcB of >60 ms from baseline. The PR interval, QRS width, QT interval, QTcB interval, QTcF interval and RR interval were not affected by the amiselimod doses.

Pharmacokinetic and safety profiles in humans

Plasma concentrations of amiselimod and amiselimod-P increased in an approximately dose-proportional manner during the 21 day dosing period, as reflected by an increase in C_{max} and AUC_{τ} over the 0.125 to 0.75 mg dose levels (Table-S2). Amiselimod reached C_{max} within 16 h on day 1 (median t_{max} 12.00–16.00 h) and within 10 h on day 21 (median t_{max} 6.00–10.00 h) of the 21 day dosing period. Amiselimod-P reached C_{max} at 12 h on day 1 (median t_{max} 12.00 h) and within 12 h on day 21 (median t_{max} 8.00–12.00 h) of the 21 day dosing period (Figure 7). A unique pharmacokinetic profile was observed for both amiselimod and amiselimod-P that showed a long elimination $t_{1/2}$. The mean $t_{1/2}$ of amiselimod ranged from 386 to 423 h. The mean $t_{1/2}$ of amiselimod-P ranged from 376 to 404 h (Table S2). Less than 0.2 and 0.05% of the administered dose was excreted in the urine on day 21 for amiselimod and amiselimod-P, respectively, suggesting that non-renal clearance including hepatic metabolism is the predominant route of elimination.

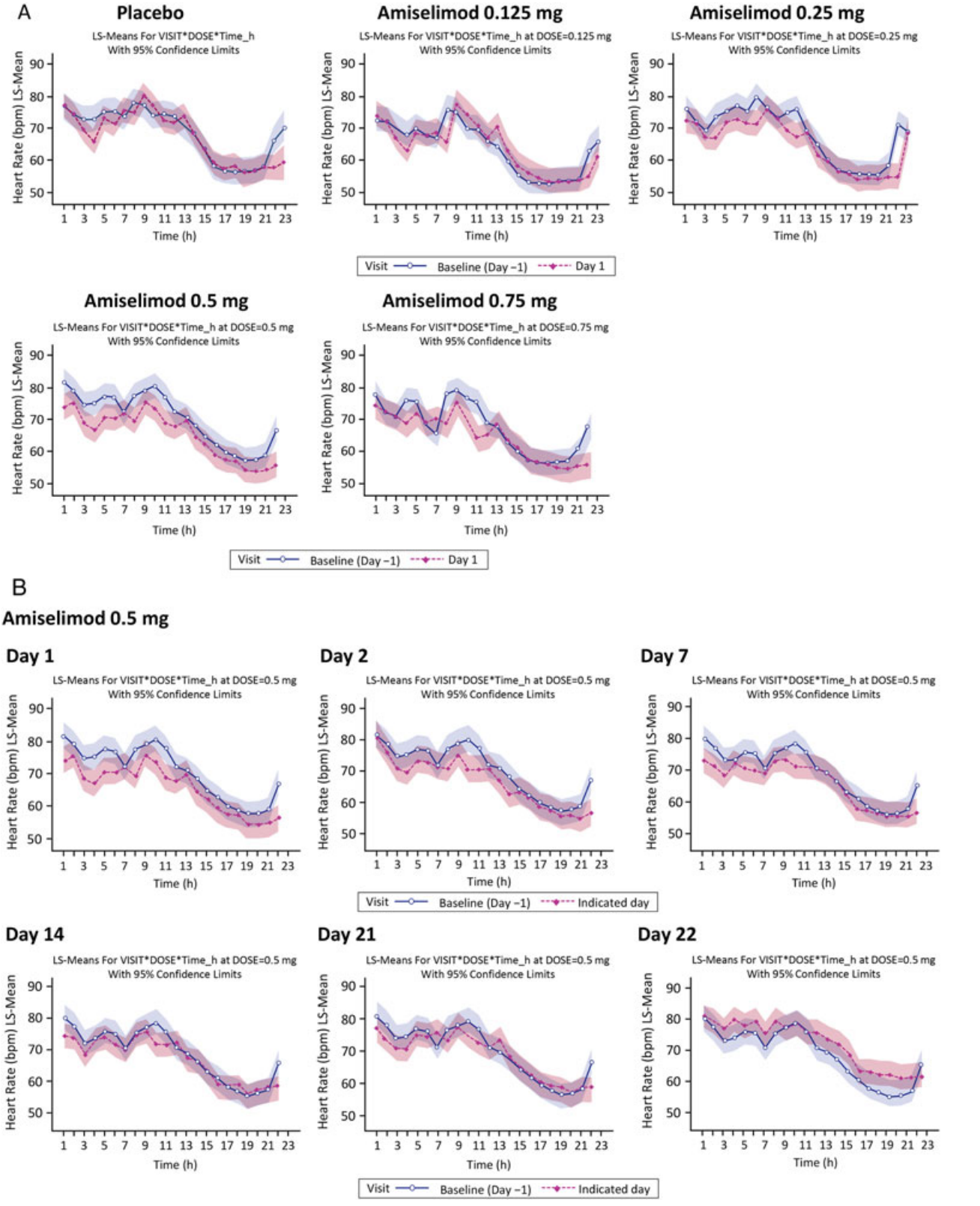

Figure 6

Mean hourly heart rate curve determined by 24 h 12-lead Holter ECG in healthy subjects. Data were analysed using a linear mixed-effect model (with 95% CI) compared with day −1. (A) Mean hourly heart rate curve on day 1 compared with day −1 in all groups. (B) Mean hourly heart rate curves on day −1 (baseline) and on days 1–22 in subjects given 0.5 mg of amiselimod. Amiselimod: $n = 10$ per dose; placebo: $n = 10$.

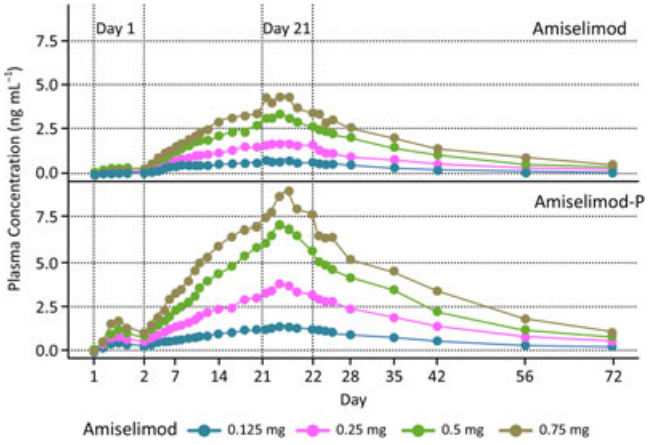

Figure 7

Mean plasma concentration–time profiles of amiselimod and amiselimod-P in healthy subjects. Blood samples were obtained on days 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 16, 18, 20, 21, 22, 23, 24, 25, 28, 35, 42, 56 and 70 after administration of amiselimod. The lower limit of quantitation was 0.05 ng·mL^{-1}. n = 10 per dose.

There were no severe AEs or serious AEs reported during the study, and there were no withdrawals because of AEs. A total of 74 AEs were reported by 28 subjects receiving amiselimod, and 16 AEs were observed in 9 subjects receiving placebo during the study. Of the reported AEs, the majority were mild, e.g. headache, and no dose relationship was found between groups. Mild/moderate AEs were reported in 8/1 subjects at 0.125 mg respectively; similarly, mild/moderate AEs were reported in 4/0 subjects at 0.25 mg, 9/1 subjects at 0.5 mg, 7/1 subjects at 0.75 mg and 8/2 subjects in the placebo group. These AEs were not suspected to be related to the study medication except for one case of flatulence in the 0.75 mg group. There were no clinically significant abnormal laboratory values, ECG parameters, telemetry, pulmonary function tests or 24 h Holter data reported, with the exception of the expected pharmacological actions on lymphocytes. In some cases, the vital signs were outside normal ranges, although no treatment or dose-related trends were observed. None of these out-of-range values was considered to be clinically significant after evaluation by the investigator.

Discussion and Conclusions

This study investigated the pharmacological profile of amiselimod with a focus on its cardiac effects. Amiselimod-P had potent selectivity for $S1P_1$ receptors and showed minimal cardiac effects in monkeys and humans, possibly because of its weak GIRK activation potential and relatively low distribution to heart, as assessed in rat heart tissues, as well as its long elimination $t_{1/2}$ in humans. The distribution of amiselimod, amiselimod-P, fingolimod and fingolimod-P in humans is unknown. However, it is generally considered that there is little difference in drug distribution between rats and humans (Sawada *et al.*, 1984; Sawada *et al.*, 1985). Therefore, amiselimod-P levels in the heart after oral administration are likely to be relatively lower than fingolimod-P in humans, as well as rats. We would conclude that amiselimod appears to be a safe drug with potent therapeutic efficacy and minimal observed cardiac effects at the anticipated clinical dose.

No significant decrease in heart rate was observed after administration of amiselimod, whereas a clinical dose (0.5 mg) of fingolimod caused a decrease in heart rate by 6.2 bpm (Schmouder *et al.*, 2012). Camm *et al.* (2014) also reported that fingolimod led to a decrease in heart rate. On the other hand, a decrease in lymphocyte count was observed after administration of amiselimod. Lymphocyte counts were decreased below the baseline by 73% with fingolimod 0.5 mg, the clinical dosage used for RRMS patients (Kappos *et al.*, 2010). Another S1P receptor modulator, ponesimod, showed significant efficacy in psoriasis patients in a Phase II trial, at 20 and 40 mg doses with a concomitant decrease in the lymphocyte count of 56 and 65% respectively (Vaclavkova *et al.*, 2014). Siponimod at 2 mg demonstrated similar efficacy to 10 mg in RRMS patients in a Phase II trial with a lymphocyte count decrease of 70% (Selmaj *et al.*, 2013), and the primary endpoint was met for 1 mg ozanimod in a Phase II trial in ulcerative colitis patients with a lymphocyte count decrease of 65% from baseline in healthy volunteers (Olson *et al.*, 2013; Sandborn *et al.*, 2015). We showed here, a lymphocyte reduction of approximately 60% after 0.5 mg amiselimod, which is expected to show efficacy in RRMS as well as other autoimmune diseases. To the best of our knowledge, this is the first S1P receptor modulator to show no significant decrease in heart rate at first administration at an estimated clinical dose, without a titration regimen. Moreover, there was no clinically significant heart rate decrease up to 0.75 mg, which is well above the currently anticipated therapeutic dose, suggesting amiselimod has a wider safety margin for heart rate reduction than other S1P receptor modulators.

The $t_{1/2}$ of amiselimod and amiselimod-P in humans is approximately 380–420 h and about twice as long as that of fingolimod (Kovarik *et al.*, 2004). In addition, the $t_{1/2}$ values of ponesimod, siponimod and ozanimod have been reported to be 21.7–33.4, 21.9–36.3 and 17.1 h, respectively, indicating that amiselimod/amiselimod-P has a $t_{1/2}$ more than 10 times longer than these direct-acting agents (Gergely *et al.*, 2012; Brooks *et al.*, 2013; Brossard *et al.*, 2013). The long $t_{1/2}$ of amiselimod and amiselimod-P in humans indicates that both will slowly accumulate to steady state over a period of approximately 10 weeks. The ranges for the accumulation ratios for amiselimod and amiselimod-P, after 21 day dosing, were approximately 16–29 and 7–9, respectively. This unique pharmacokinetic profile therefore may be advantageous when initiating amiselimod treatment, as low initial doses of amiselimod reduce the potential for bradycardia, and desensitization can be expected to occur gradually over several weeks of accumulation, rendering dose titration unnecessary. Moreover, our studies revealed that, after oral administration, amiselimod-P may be distributed at lower levels in human heart tissue and in rats when compared with fingolimod-P. The pharmacokinetic profile of amiselimod may explain the lack of bradycardia at first dose.

The fingolimod-induced AV conduction block may be a result of a direct effect of the drug on intrinsic AV nodal electrophysiology, as well as an activation of GIRK channels

via S1P receptors (Egom *et al.*, 2015). However, fingolimod or fingolimod-P did not affect cardiac Na^+ and Ca^{2+} channels in HCMs (data not shown), suggesting that fingolimod-induced AV conduction block is mediated via the activation of GIRK channels, which were reported to induce AV conduction block (Drici *et al.*, 2000). The amiselimod profiles reported in the current study suggest a low risk for the induction of AV conduction block as well as heart rate reduction. Indeed, no AV conduction block of clinical concern was detected in the amiselimod groups in this clinical study.

Amiselimod-P resulted in an approximately five-fold weaker activation of GIRK channels in human primary atrial myocytes compared with fingolimod-P. Because the $S1P_1$ receptor agonist activity of amiselimod-P was similar to that of fingolimod-P, the potential for bradycardia caused by amiselimod-P is likely to be less than that for fingolimod-P, owing to its weaker activation of GIRK channels. The precise mechanism(s) underlying the difference in GIRK activation remains to be elucidated. However, it can be speculated that there may be a difference between amiselimod-P and fingolimod-P regarding the strength of GIRK channel activation, related to the signalling downstream of $S1P_1$ receptors.

Similar to fingolimod (Paugh *et al.*, 2003), amiselimod is predominantly phosphorylated by SPHK-2 (unpublished data). Levels of *SPHK-2* mRNA are highest in the kidney and liver in humans and its expression has been detected in the heart (Liu *et al.*, 2000). Amiselimod-P and fingolimod-P were detected in supernatants when added to primary HCMs. Therefore, a local conversion to amiselimod-P might occur in the heart and might contribute, in part, to amiselimod-P concentrations in the heart. The observation that amiselimod-P concentrations in rat hearts were lower than those of fingolimod-P after administration of amiselimod or fingolimod might be explained by a slower local conversion to amiselimod-P in the heart than to fingolimod-P, as well as a difference in tissue distribution between these compounds. In contrast, our preliminary study indicated the rate of phosphorylation of amiselimod by SPHK-2 was similar to that of fingolimod, indicating that a different mechanism of action underlies the slower conversion rate of amiselimod at the cellular level, compared with fingolimod.

Our study has some limitations that should be considered before making precise quantitation of the effect on heart rate. First, as this multiple ascending-dose study was primarily designed to assess pharmacokinetics and tolerability of amiselimod, the protocol did not include attempts to reduce heart rate variability or maintain similar heart rates in the subjects before its administration. Second, while amiselimod did not significantly affect the heart rate in any dose group on day 1, there were differences in baseline data on day −1. Third, the heart rate was strongly affected by the conditions of the individual subjects and showed a large inter-individual variation during the study. Fourth, this study was not designed to have sufficient statistical power to detect differences in the nadir mean hourly heart rate between groups. Therefore, we have planned another clinical pharmacology study specifically designed to clarify the relationship between heart rate decrease and drug exposure and to reduce the variation in heart rate by multiple ascending doses without titration.

No clinically significant negative chronotropic effects were observed up to the 0.75 mg dose in healthy volunteers in this study. The mechanism of action underlying the lack of bradycardia observed upon the administration of amiselimod to humans is not well understood. However, its weak GIRK activation potential and its unique pharmacokinetic profile including low distribution to heart and long elimination $t_{1/2}$ are likely to contribute to the lack of bradycardia at first dose. A randomized, double-blind, placebo-controlled Phase II trial reported that treatment with amiselimod at doses of 0.2 and 0.4 mg had a significant therapeutic effect on MRI parameters in patients with RRMS, without clinically significant heart rate reduction (Kappos *et al.*, 2016). Therefore, it is expected that amiselimod could have potent therapeutic efficacy in RRMS, as well as other autoimmune diseases including inflammatory bowel disease, while minimizing effects on heart rate without requiring dose titration.

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Author contributions

K.S., H.K., M.A. and T.Y. designed the nonclinical studies. Y. M., K.S., A.M., H.K., K.O. and K.H. performed the nonclinical studies. K.S., A.M., H.K., K.O. and M.A. analysed the data. A. K., T.H. and P.N. designed the clinical studies, and P.N. and S.I. analysed the clinical studies. K.S. and A.K. wrote the manuscript.

Conflict of interest

K. Sugahara, Y. Maeda, K. Shimano, A. Mogami, H. Kataoka, K. Ogawa, K. Hikida, H. Kumagai, M. Asayama, T. Yamamoto, S. Inoue and A. Kawaguchi are employees of Mitsubishi Tanabe Pharma Corporation, Japan. T. Harada and P. Ni are employees of Mitsubishi Tanabe Pharma Europe.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organizations engaged with supporting research.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.13641

Figure S1 Effects of amiselimod-P, fingolimod-P, and S1P on human GIRK currents. GIRK currents recorded in human atrial myocytes were measured using the whole-cell patch clamp method. Representative trace at −100 mV illustrating the effects of S1P (A), fingolimod-P (B), and amiselimod-P (C) on GIRK currents.

Figure S2 Effect of amiselimod on heart rate in cynomolgus monkeys. Amiselimod was orally administered to conscious male cynomolgus monkeys in a dose ascending manner at dose levels of vehicle, 0.3, 3 and 30 mg kg^{-1}, with a 6-day interval between vehicle and 0.3 mg kg^{-1}, and 13- or 14-day intervals between dose levels of 0.3, 3 and 30 mg kg^{-1}. Heart rate and ECG were analysed before and at 4, 8, 24, 48 and 72 h after each dose using the telemetry system. Results are expressed as the mean ± SD (n = 4).

Figure S3 Effects of 0.75 mg of amiselimod on mean hourly heart rate in healthy human subjects. Mean hourly heart rate was measured by 24-h 12-lead Holter ECG. Curves on day 1 to day 21 are shown as pink bands, and the curve on day −1 as the blue band. Data were analysed using the linear mixed effect model and 95% confidence intervals are shown as the shaded area.

Table S1 Pharmacokinetic parameters of amiselimod/amiselimod-P or fingolimod/fingolimod-P after a single oral administration of amiselimod or fingolimod to rats.

Table S2 (a) Summary of amiselimod derived pharmacokinetic parameters on day 1 and day 21 (b) Summary of amiselimod-P derived pharmacokinetic parameters on day 1 and day 21.